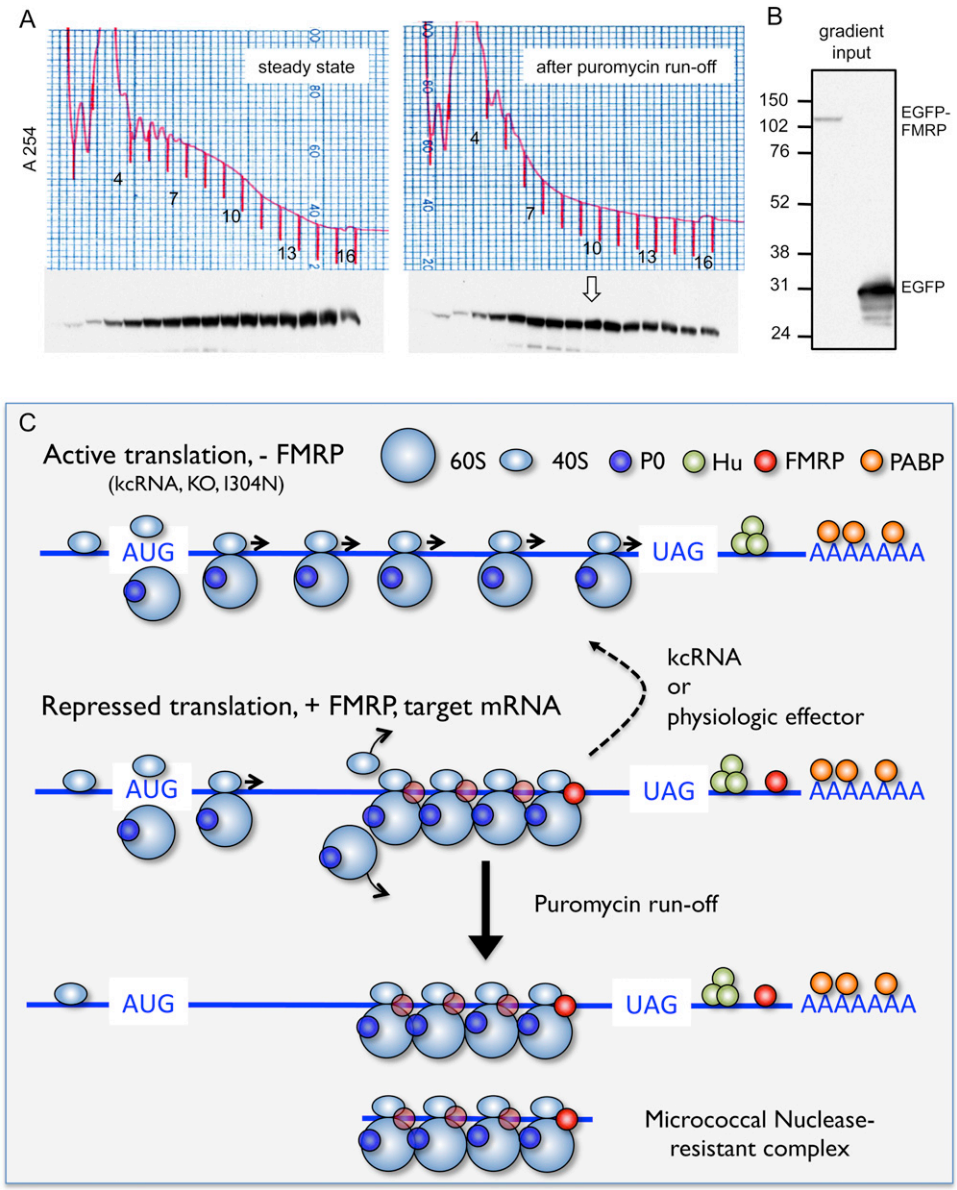

Figure S7. Immunogold Electron Microscopic Analysis and Model for the Action of FMRP as a Translational Inhibitor, Related to Figure 7

(A) For immuno-EM analysis, EGFP-tagged FMRP or EGFP alone was expressed in 293T cells for 48 hr followed by treatment with 1 mM puromycin or vehicle for 1 hr followed by 10 min in 0.1 mg/ml CHX. Post mitochondrial cell extracts were analyzed by sucrose gradient density analysis with monitoring at A254. A254 traces of EGFP-FMRP transfected cells without (steady state) or with puromycin (puro. runoff) are shown to illustrate the extent of translocating ribosome run-off. Western blot analysis of these gradient fractions with ab17722 shows FMRP present in the heaviest polyribosomal fractions in the steady state and a characteristic shift to the stalled complex fractions after puromycin run-off in vivo. The fraction used for immuno-EM (Figure 7C) is marked with a white arrow. The same fraction from gradients from cells expressing EGFP alone was also analyze by immuno-EM in parallel.

(B) Aliquots of postmitochondrial lysate (S2) input to the sucrose gradients were analyzed by Western blot with anti-EGFP antibody to confirm EGFP expression as a negative control for the immuno-EM studies.

(C) Model of FMRP action to stall ribosomal elongation. Upper panel, Active translation: In the absence of FMRP, brain transcripts are translated into protein by translocating ribosomes (made up of 40S and 60S subunits shown in light blue) which assemble at the start codon (AUG, initiation) and dissociate at the stop codon (i.e., UAG, termination). Ribosomal protein P0 is shown as a darker blue sphere on the 60S subunits. The poly(A) binding protein PABP and the Hu family of RNABPs interacting with specific binding sites in 3′UTRs are depicted with orange and green spheres, respectively. All 4 of these RNA binding proteins are polyribosome-associated, each by a different mechanism, and for this reason PABP, Hu and P0 are used throughout this work as controls for the properties and function of FMRP. Middle panel, repressed translation associated with FMRP interaction with target mRNAs. FMRP preferentially interacts with specific mRNAs (Table S2) and in this context inhibits protein synthesis by stalling ribosomal translocation on those transcripts, as evidenced by their puromycin insensitivity (lower panels of figure, see also Figures 3, Figure 4, Figure 5, and Figure 6). This inhibition is reversible, as it can be acutely relieved by competing FMRP off of

polyribosomes with kcRNA decoy (dotted arrow); it is unknown whether this might occur in vivo by the phosphorylation state of FMRP, its degradation by the proteasome, interactions with other proteins, non-coding RNAs or other physiologic effectors.

Hu and PABP are not removed from mRNAs by puromycin run-off. However, since Hu and PABP are predominantly associated with transcripts that do not harbor puromycin-insensitive ribosomes (a different or much broader population than those bound by FMRP), they shift more completely in puromycin run-off experiments (Figure 3B-C). The extent to which they do not fully run-off with puromycin treatment may indicate some overlap with the set of translationally repressed mRNAs. Likewise, the robust run-off seen with rpP0 demonstrates that the vast majority of ribosomes are not stalled but dissociate readily with puromycin as they translocate. Lower panel, micrococcal nuclease-resistant complex: FMRP inhibits ribosomal translocation in a complex consisting of target mRNA and several stacked or condensed ribosomes, as evidenced by its micrococcal nuclease resistance and CLIP results on the stalled complexes (see also Figure 7). The stoichiometry of FMRP and stalled ribosomes remains to be determined. We have drawn a minimum of one (red) FMRP present in the stalled complex, recognizing the possibility that additional FMRP molecules (illustrated by transparent red figures) may be present. The presence of some FMRP in the UTRs (depicted on the 3′UTR) is consistent with the release of some FMRP by MN treatment. We have shown that, in the steady-state, approximately equal numbers of ribosomes are present on FMRP-target transcripts in the presence or absence of FMRP (see Figure 4, first column). In addition, we find that some of the ribosomes on FMRP-stalled transcripts are puromycin sensitive (Figures 3–6). One possible explanation for these observations is that in the presence of the stalled complex on the CDS, ribosomes continue to initiate, but drop off (Nottrott et al., 2006; Petersen et al., 2006) upon encountering the stalled complex, as depicted in the "repressed translation" state.